also are responsible for undesirable bitterness, astringency, and haze formation in fruit juices and wines (1,4). Because of these properties, convenient and accurate methods for the analysis of phenolic compounds are needed to study and control their effects on the quality of fruit and vegetable products.

Phenolic compounds in plant tissues have been determined by a number of analytical procedures including spectrophotometry (5,6), gas-liquid chromatography (GLC) (7,8), and HPLC (4,9,10). Malmberg and Theander (11) reported good agreement of data obtained by GLC and HPLC for the content of chlorogenic acid in potatoes, but data obtained spectrophotometrically had only fair agreement with that of the other two methods. Recently, HPLC has become quite popular due to advances in HPLC column design and ease of analysis. Unlike in GLC analyses compounds of high molecular weight can be analyzed by HPLC without derivatization, and analysis of many components of biological samples frequently can be carried out on crude extracts. Furthermore, HPLC is applicable to the selective analysis of specific individual compounds rather than broad classes of compounds as is the case with many spectrophotometric methods. Unfortunately, the commonly-used methods employing single wavelength HPLC detection can lead to incorrect assumptions about peak identity and, in turn, to faulty quantification of phenolic compounds in plant tissues. Recently, Law et al. (12) showed that the use of dual-wavelength UV detection techniques allowed more accurate identification and quantification of plant flavonoids with HPLC.

In this report, we describe an HPLC system for the separation of nine phenolic acids and related compounds that are commonly found in plant materials. Extracts containing the phenolic components of apple tissue were separated by this system, and individual compounds were identified and quantified, when possible, with dual wavelength detection and absorbance ratio measurements.

MATERIALS AND METHODS

Standards

Nine phenolic compounds were selected as standards based on reports of phenolic composition in apples (4,5) and on our own preliminary work. The

authentic compounds were purchased from Sigma Chemical Co., (St. Louis, MO). Analytical HPLC data and UV spectra were obtained from methanolic solutions of the authentic compounds.

Instrumentation

Separations were carried out with a Waters HPLC system (Waters Associates, Milford, MA) consisting of two Model 600A pumps, a Model 660 solvent programmer, a Model 490 programmable multiwavelength detector, a Model 7125 sample injector (Rheodyne Inc., Cotati, CA), and a 4.6 mm i.d. by 25 cm Econosil C-18 (particle size, 5 μm) column (Alltech Associates, Inc., Deerfield, IL), in combination with a Model SP4200, two-channel computing integrator (Spectra-Physics, San Jose, CA). A Waters Guard-Pak precolumn was installed at the head of the analytical column. Absorbance ratio responses were obtained from a two-channel Omniscribe strip chart recorder (Houston Instruments, Inc., Austin, TX).

Chromatographic Procedures

Two solvents were used: 5% (V/V) aqueous acetic acid (J. T. Baker, Chemical Co., Phillipsburg, NJ) and spectral grade acetonitrile (Burdick and Jackson, Muskegon, MI); both solvents were filtered through 0.45 μm membrane filters. Solvents were degassed continuously with helium. During an analysis, the solvent gradient was programmed from 12% to 20% acetonitrile in 10 minutes with a built-in program (curve 9) of the Model 660 programmer. The flow rate was set at 1.5 ml/min. Column pressure was generally about 2500 psi.

Sample Preparation

Apples (*Malus sylvestris* cv. Golden Delicious) were obtained during the month of October from a local food store. Cylindrical plugs of about 10 g each were taken with a cork borer from the surface toward the core of apples. The plugs (skin removed) were weighed, cut into pieces, added to a stainless steel, semimicro blending jar containing 100 ml of 80% (by volume) methanol in water and blended for 2 minutes at high speed. The homogenate was decanted into a 200 ml volumetric flask, the blender jar was rinsed with additional portions of

80% methanol, and the rinsings were added to the flask to bring the volume to a total of 200 ml. Two grams of Celite analytical filter aid were added to the flask, the contents were mixed and then filtered through a Whatman No. 541 filter paper in a Buchner funnel under vacuum. An aliquot consisting of 40 ml of the extract, which represented 2 g of fresh sample, was passed through a 1.2 cm i.d. by 6.5 cm disposable extraction column (J. T. Baker Chemical Co., Phillipsburg, NJ) packed with a 1 cm bed of C-18 HPLC packing for the purpose of removing lipids and other non-polar components that otherwise would have been strongly retained by the analytical HPLC column. The solvent was removed under vacuum at 35° C, and the residue was made up to 2 ml with methanol and filtered through a 0.45 μ membrane filter. A 20 μl aliquot of the sample solution was analyzed by HPLC, and the content of phenolic compounds was reported as mg of compound per kg fresh weight of apple tissue.

Quantitation

Calibration of retention times, response factors, and absorbance ratios was carried out with a standard mixture containing 1.111 μg per injection of the nine phenolic compounds listed in Table 1. Detector response factors were determined for authentic compounds at wavelengths of 254 and 280 nm with chlorogenic acid serving as a reference peak. Chromatograms of the authentic compounds or the components of apple extract and the resulting calculations were handled sequentially for 280 and 254 nm wavelengths on channels A and B of the integrator. At the time of detection, two different absorbance ratios (A_{284}/A_{272} and A_{265}/A_{272}) were recorded by the two pens of the recorder.

RESULTS AND DISCUSSION

Data Obtained with Standards

The nine phenolic compounds that were used as standards are listed in the order of their elution in Table 1. Two pairs of compounds; p-hydroxybenzoic acid and (-)-epicatechin, and ferulic- and sinapic acids; have very close retention times. As an additional complication, complex mixtures from natural

TABLE 1

Retention Times, Capacity Factors, Relative Retention, and Absorbance Ratios of Authentic Phenolic Compounds

Pk. No.	Compound	Ret. time (min)	Capacity factor (K')	Relative retention (α)	λ max (nm)	Absorbance ratio 265/272nm	Absorbance ratio 284/272nm
1.	Protocatechuic acid	3.98	0.81		258	1.70	0.86
				1.52			
2.	(+)-Catechin	4.90	1.23		276	0.65	0.91
				1.28			
3.	Chlorogenic acid	5.67	1.58		325	0.66	1.90
				1.32			
4.	p-Hydroxybenzoic acid	6.77	2.08		255	1.56	0.16
				1.13			
5.	(-)-Epicatechin	7.36	2.35		276	0.36	0.85
				1.18			
6.	Caffeic acid	8.32	2.78		322	0.71	1.74
				1.92			
7.	p-Coumaric acid	13.92	5.33		310	0.60	1.74
				1.15			
8.	Ferulic acid	15.64	6.11		318	0.65	1.80
				1.03			
9.	Sinapic acid	16.09	6.31		322	0.70	2.16

$$K' = \frac{t_R - t_0}{t_0} \quad ; \quad (t_0 = 2.2)$$

$$\alpha' = \frac{k'_2}{k'_1}$$

sources often contain other compounds that co-elute from HPLC columns with compounds of interest and make reliable quantification difficult. For these reasons, we used detection at two wavelengths and comparison of absorbance ratios to avoid inaccurate analyses.

Others have used absorbance ratios obtained with two or more wavelengths to identify or determine the purity of various compounds (12-15). The phenol: compounds in our study were chemically diverse with corresponding diversity o: absorbance spectra. We found that absorbance ratio measurements with wavelen: of 272 nm and either 265 or 284 nm (absorbance at 272 nm was the divisor and absorbance at one of the other two wavelengths was the dividend) represented the best compromise, allowing us to distinguish between the phenolic compounds

of interest in this study. The values of the absorbance ratios obtained with the HPLC system described above for nine authentic phenolic compounds are presented in Table 1 and are in close agreement with values obtained by simple spectrophotometric analysis. Chromatograms of the separated mixture of authentic compounds detected at 254 nm and 280 nm are presented in Figures 1 and 2, respectively. Machine-plotted ratios of absorbance at wavelengths of 265/272 nm and 284/272 nm are shown in Figures 1 and 2 below the chromatograms. By virtue of their symmetrical rectangular geometry, the machine-plotted absorbance ratios reflect the high purity of the individual compounds.

To test the accuracy of the overall method described in Sample Preparation, duplicate aliquots of a pre-analyzed sample of apple extract containing 0.656 µg of indigenous chlorogenic acid per 20 µl injection were spiked with authentic chlorogenic acid to contain added amounts of 1.125, 2.500 and 5.000 µg per 20 µl injection. Recoveries of 98% to 100%, were obtained for the added chlorogenic acid. Hence, negligable sample is lost in the clean-up procedure.

HPLC Analysis of Phenolic Components of Apple

Samples of Golden Delicious apples were analyzed for phenolic compounds, and the HPLC chromatograms and the corresponding plots of absorbance ratios are shown in Figures 3 and 4. Separated compounds of the apple extract that had retention times equivalent to those of components of the mixture of authentic compounds were assigned numbers that correspond to those of the authentic compounds listed in Table 1 and shown in Figures 1 and 2. Calculation of the content of these compounds in apples was made for the components of the extract that had retention times coincident with authentic compounds and was based on recovery data and recorder responses established for authentic compounds. This data is presented in Table 2 along with absorbance ratios for each of the numbered components. However, as will be discussed, the absorbance ratios demonstrated in several cases the well-recognized faultiness of the assumption that coincidence of retention time is a sound basis for identification from which qualitative data can follow.

Several generalizations can be drawn from the data shown in Table 2. First, chlorogenic acid is a major component in the extracts. This agrees

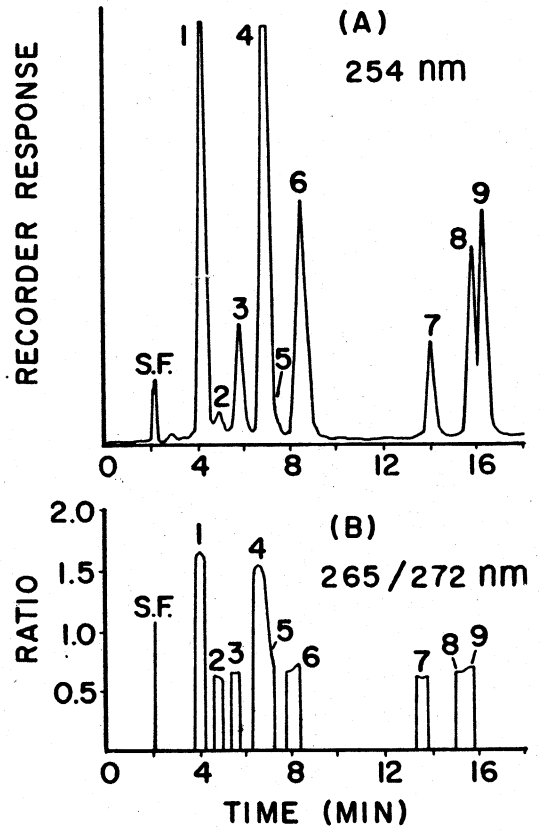

Figure 1. (A) HPLC chromatogram of authentic phenolic compounds detected at a wavelength of 254 nm. Peak numbers correspond to compound names listed in Table 1. S.F. = solvent front. (B) Absorbance ratios for authentic phenolic compounds detected: $A_{265nm} \div A_{272nm}$.

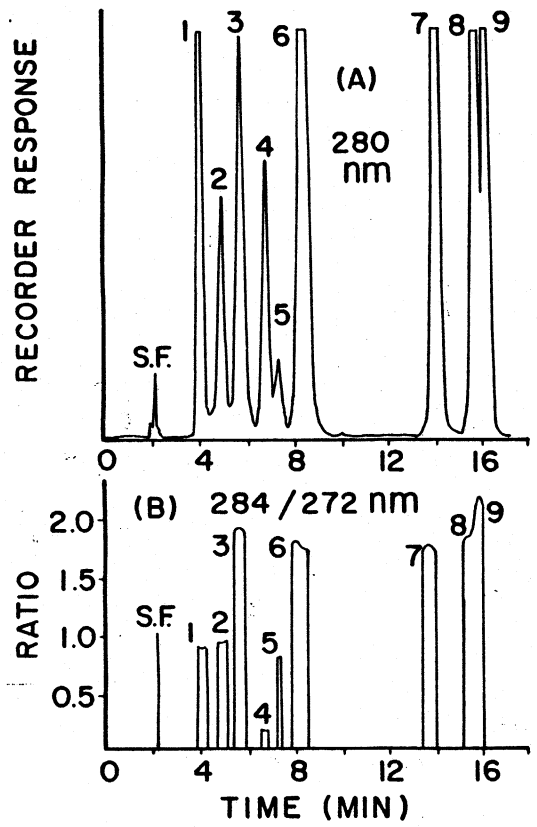

Figure 2. (A) HPLC chromatogram of authentic phenolic compounds detected at a wavelength of 280 nm. Peak numbers correspond to compound names listed in Table 1. S.F. = solvent front. (B) Absorbance ratios for authentic phenolic compounds detected: $A_{284nm} \div A_{272nm}$.

TABLE 2

Content of Phenolic Compounds in Golden Delicious Apples

Compound	Concentration in apple tissue (mg/kg fresh weight) -a-		Absorbance ratio	
	254 nm	280 nm	265/272 nm	284/272 nm
Catechin	18.0 ± 3.5	10.0 ± 2.5	0.78 ± 0.02[b]	0.98 ± 0.0
Chlorogenic acid	78.5 ± 4.5	81.0 ± 3.5	0.65 ± 0.00	1.80 ± 0.0
p-Hydroxybenzoic acid	4.5 ± 1.5	31.0 ± 4.0	0.82 ± 0.00	1.60 ± 0.0
Epicatechin	-c-	78.5 ± 12.0	0.65 ± 0.00	0.95 ± 0.0
P-Coumaric acid	7.5 ± 1.0	2.5 ± 0.5	0.77 ± 0.00[b]	1.00 ± 0.0
Ferulic acid	2.0	0.5[b] ± 4.0	-e-	-e-
Sinapic acid	2.5 ± 0.5	9.0 ± 4.0	0.70 ± 0.00[b]	1.27 ± 0.0

-a- Mean for triplicate determination; standard deviation indicated with ± sign

-b- Two determinations made; data presented in table is the mean value; the observed deviation from the mean is shown after the ± sign.

-c- Epicatechin was not determined at 254 nm because it has a low extinction coefficient at this wavelength and was not completely resolved from other components in the mixture.

-d- Only one determination made.

-e- Not calculated due to the low concentration of ferulic acid in the sample.

substantially with Lea (8) who reported that chlorogenic acid was one of the principal phenolic components of apple flesh. The accuracy of the analytical identity and quantity of this compound is evident, due to the agreement of both absorbance ratios and of the closeness of concentration determinations of chlorogenic acid at the two different wavelengths. Inspection of the data for the minor components, however, reveals that great care must be taken in its interpretation. For example, there is a large disparity between the concentration of the component with the retention time of p-hydroxybenzoic acid determir at 254 and 280 nm. The absorbance ratios obtained from the chromatogram reveal extreme heterogeneity of the material that this "peak" represents (note, for example, the ratio of absorbance at 284 and 272 nm). The quantitative value

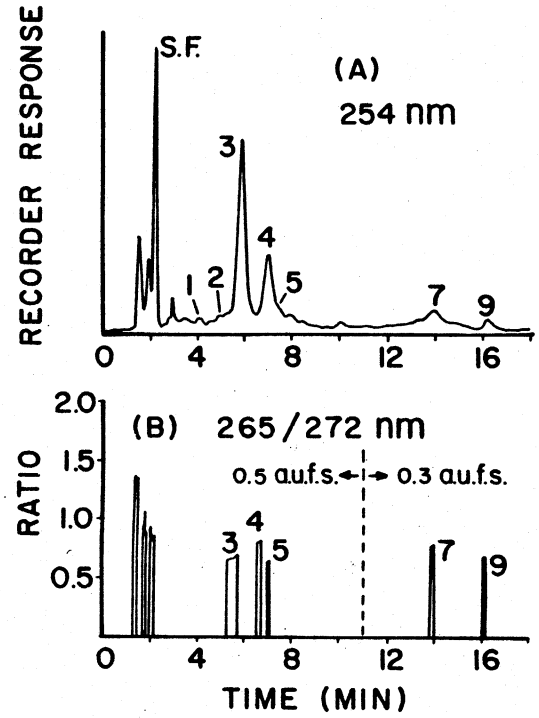

Figure 3. (A) HPLC chromatogram of extract of Golden Delicious apples detected at a wavelength of 254 nm. S.F. = solvent front. (B) Absorbance ratios for components detected: $A_{265nm} \div A_{272nm}$. (a.u.f.s. = absorbance units full scale).

obtained at 254 nm probably more closely reflects the actual value for the amount of p-hydroxybenzoic acid in this component, since that wavelength is closer to the absorption maximum for p-hydroxybenzoic acid.

The levels of catechin determined at 254 and 280 nm are different, but fall nearly within the range of experimental error of each other. The absorbance ratios for this component also are quite similar to those given for the standard compound. A reasonable estimate of the level of this compound may be the average of the two values.

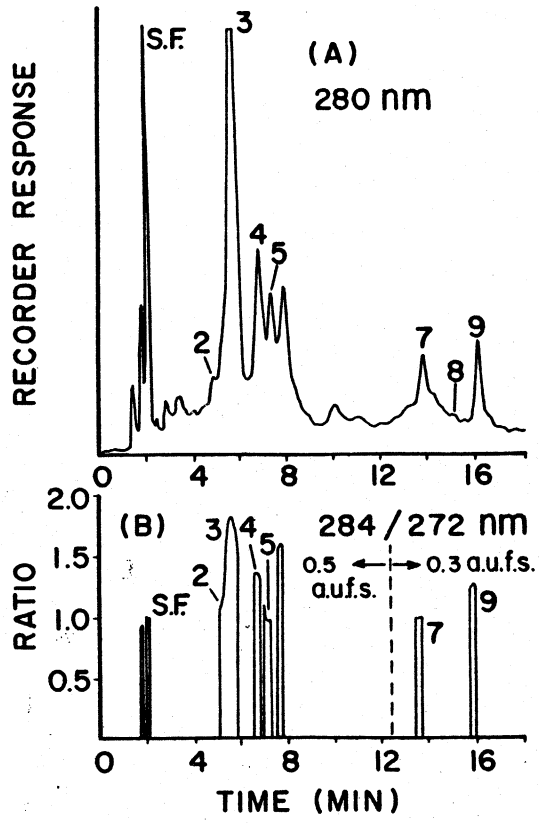

Figure 4. (A) HPLC chromatogram of extract of Golden Delicious apples detec at a wavelength of 280 nm. S.F. = solvent front. (B) Absorbance ratios for components detected: $A_{284nm} \div A_{272nm}$. (a.u.f.s. = absorbance units full scale).

The identity of the component with the proper retention time for epicat is supported by the absorbance ratio at 284/272 nm, but the identity and concentration are not well-supported by the other data. To some extent this may be due to the low extinction coefficient of epicatechin at lower wavele (i.e., 254 and 265 nm).

A minor component with the proper retention time for protocatechuic acid was noted in the chromatogram shown in Figure 3. However, this component was not present in sufficient quantity to obtain absorbance ratios and consequently no data was collected or tabulated for this unknown.

The remaining compounds in Table 2, coumaric-, ferulic- and sinapic acids, were all very minor components in the mixture. None of these compounds appear to give either the theoretical values for absorbance ratios or good agreement between quantitative values at both wavelengths. Hence, it is not possible to derive information about their levels apart from the obvious fact that they are not very abundant in this variety of apple.

The number of phenolic compounds isolated from apple tissue that we were able to identify and quantify with confidence is less than reported by other authors (4,6). However the use of absorbance ratios caused us to exclude components that would have been accepted if we had based identities and homogeneity of components only on retention time data.

Separation, identification and quantitation of the phenolic compounds of plant tissues are difficult challenges, but all three can be improved with better HPLC separations, dual wavelength detection and absorbance ratio comparisons. Our experience suggests that data in the literature that stemmed from HPLC quantification of plant metabolites at a single wavelength should be viewed with suspicion.

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